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*Fee*

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29295/BN

**DNA construct and its use.**

The present invention relates to a new DNA construct for transformation into oilseed plants. The DNA construct comprises nucleotide sequences encoding peptides with enzyme activities necessary for the high-level production and esterification of keto group-containing xanthophylls in oilseed plants.

**Background of the invention**

Carotenoids are produced *de novo* by plants, fungi, algae and some bacteria. A number of biosynthetic steps are needed for the biological production of the carotenoids. There are two chemically different groups of carotenoids, namely carotenes containing only carbon and hydrogen molecules and xanthophylls containing oxygen in the molecule in addition to carbon and hydrogen.

The xanthophylls, and particularly astaxanthin (3,3'-dihydroxy-β-β-carotene-4,4'-dione), are often colored pigments and are used as such or as anti-oxidants.

Carotenes are biological precursors for the production of the oxygen-containing xanthophylls. There are two types of enzymes responsible for the introduction of hydroxy groups and keto groups into the carotenes, namely hydroxylases and ketolases, respectively.

The keto group-containing xanthophyll astaxanthin, which has keto and hydroxy groups, is biosynthetically produced from beta-carotene.

Large-scale production of xanthophylles from natural sources is at present performed by AstaCarotene AB, Gustavsberg, Sweden, by cultivation of the alga *Haematococcus pluvialis* for the production of astaxanthin in esterified form.

It would be desirable to be able to produce keto group-containing xanthophylls particularly astaxanthin, in oilseed plants. Oilseed plants have naturally β-carotene hydroxylases but lack β-carotene C-4-oxygenase enzymes or ketolases.

**Description of the invention**

The present invention provides DNA constructs enabling and promoting production of keto group containing xanthophylls, especially astaxanthin, in oilseed plants, such as rape, sunflower, soybean and mustard. The DNA construct is transformed into the oilseed plant cell for expression of a protein or fused protein which has an enzyme activity enabling keto group insertion into a carotene or hydroxy carotene for the biosynthetic production of a keto group containing xanthophyll, such as cantaxanthin (β,β-carotene-4,4'-dione) and/or astaxanthin. Use is thus made of the biosynthetic pathway of the oilseed plant to

produce carotenoids. The naturally occurring synthesis of carotenoids involves a number of enzymes, namely 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, and β-carotene C-4-oxygenase. Genes coding for peptides having these enzymatic activities may be inserted into the DNA construct of the invention, one or several per construct, to promote high-level production in the transgenic oilseed plant. In case only one enzyme coding gene is inserted per plant, two or more plants may be sexually interbred to produce plants containing all the desired enzyme activities.

Thus, the present invention is directed to a DNA construct comprising in the 5′ to 3′ direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

In a preferred embodiment of the invention the DNA construct additionally comprises between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

The DNA construct is preferably such that the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production is selected from the group consisting of peptides with 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, and β-carotene C-4-oxygenase activity. To promote esterification of astaxanthin a nucleotide sequence coding for a peptide with acyl transferase activity may be included in the group.

In a preferred embodiment of the DNA construct according to the invention the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β-carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

An example of the DNA construct of the invention is presented in the sequence listing as SEQ ID NO:1 and in Fig.1.

The present invention is also directed to a transgenic oilseed plant cell comprising the DNA construct of the invention, and preferably the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

The invention is additionally directed to transgenic oilseed plant-produced xanthophyll, e.g. canthaxanthin and astaxanthin.

A preferred aspect of the invention is directed to transgenic oilseed plant-produced astaxanthin esters.

The present invention will now be illustrated with reference to the DNA construct disclosed in the sequence listing and in Fig.1, and the following description of embodiments. However, the invention is not limited to these exemplifications.

**Short description of the drawings**

Fig.1 illustrates the nucleotide sequence of the DNA construct comprising the napin promoter, the chloroplast localization signal, the N-terminally truncated β-carotene C-4-oxygenase gene and the termination sequence, and the deduced amino acid sequences of the transit peptide and the β-carotene C-4-oxygenase.

**Description of embodiments**

The invention is illustrated by production of astaxanthin in the seed of oilseed rape. The astaxanthin produced in the seed of the transgenic plant is extracted as part of the extracted oil. By use of conventionally used protocols for Agrobacterium tumefaciens mediated transformation such as described by (Hoekema et al.1983, An et al. 1986, Fry et al. 1987, DeBlock et al. 1988, Radke et al.1988, or Moloney et al. 1989) transgenic plants are produced having a chimeric DNA construct that is genetically inherited and is able to produce astaxanthin. The nucleotide sequence of the chimeric DNA construct consist of four parts of different genetic origin namely: (1) a promoter, (2) a localization signal, (3) a β-carotene C-4-oxygenase coding region and (4) a termination sequence.

The napin promoter directs transcription to the seed of oilseed rape (Stålberg et al 1996). This promoter was coupled to a localization signal similar but not identical to a transit peptide (TP) of Rbcs1a (Krebbers, 1988) that directs the translated product of a fused gene to the chloroplast. The promoter and the TP sequence were ligated to a part of the coding sequence of a ketolase gene BCK (Kajiwara et al. 1995). This enzyme oxygenates β-carotene to canthaxanthin, (Fraser et al. 1997). The chimeric DNA construct was then coupled to a suitable termination sequence, e.g. that of the Agrobacterium tumefaciens nopaline synthase gene (the nos 3′ end)(Bevan et al. 1983), as illustrated in Fig.1.

Cellular storage of Astaxantin

The storage of large amounts of free astaxanthin in plants will be difficult due to toxic effects of the molecule as it intercalates in the plant membranes. An effective esterification of astaxanthin to fatty acids enables storage of the esterified molecules in triacylglycerol containing oleosomes. Thus, an acyl transferase can be claimed to be of fundamental importance for the process, as is proteins that can mediate transport of different forms of astaxanthin from the chloroplast to the vesicles.

Sequences and oligonucleotides used in the construction of the DNA construct

*1. Napin promoter (GeneBank ACCESSION No. J02798)*

This promoter sequence, a 1145 base pair fragment including the 5' leader sequence has a unique HindIII site at the 5' end. The 3' end was synthesized with an additionally 6 nucleotide BamHI site.

*2. Transit peptide similar to RBCS1a (GeneBank ACCESSION No. X13611, X14565)*

The transit peptide (TP) was amplified by PCR from –28 to the end of the transit cleavage aa=54/55 site of the Rbcs1a gene. The 5' end was synthesized with a BamHI site and similarly the 3' sequence was synthesized with a XbaI site. The two following oligonucleotides were used for the PCR amplification.

BamHI

5' primer: TP1 5'AGAC **GGATCC** TCAGTCACACAAAGAGTA 3'

SacI XbaI

3' primer: TP2 5'GTTC **GAGCTC TCTAGA** CATGCAGTTAACGC 3'

*3. BCK (β-carotene C-4 oxygenase) (Genebank ACCESSION No. D45881)*

The BCK fragment was amplified by PCR including a 5' XbaI site and was ligated to the TP already described. The 5' primer (BCK1) used for PCR, is homologous to the BCK sequence from nucleotide 264 and the 3' oligonucleotide (Ax40) ends with a stop codon and was synthesized with a SacI restriction site for cloning. The synthesized fragment was fused to the TP as shown in Fig 1.

Oligonucleotides used for PCR:

XbaI

5' primer: BCK1 5'ACAG **TCTAGA** ATGCCATCCGAGTCGTCA 3'

SacI

3'primer: AX40 5'CACC**GAGCTC**CATGACACTCTTGTGCAGA 3'

**Description f SEQ ID NO:1 and SEQ ID NO:2**

The sequences shown i Fig.1 are the same as the two sequences which are shown in the sequence listing.

The SEQ ID NO:1 is a nucleotide sequence composed of the following features:

| | Nucleotide No. |
|---|---|
| Cloning site HindIII | 1-6 |
| Napin Promoter | 1–1145 |
| Cloning site BamHI | 1146-1151 |
| Transit peptide leader | 1152-1178 |
| Transit peptide coding | 1179-1347 |
| Cloning site XbaI | 1348-1353 |
| β-carotene C-4-oxygenase | 1354-2217 |
| β-carotene C-4-oxygense 3' untranslated | 2218-2266 |
| Cloning site SacI | 2267-2272 |
| Nopaline synthetase termination | 2273-2536 |
| Cloning site EcoRI | 2538-2543 |

The SEQ ID NO: 2 is a deduced amino acid sequence of the fusion protein of the transit peptide and the peptide with β-carotene C-4-oxygenase activity.

**References**

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Radke SE, Andrews BM, Moloney MM, Crouch ML, Kridl JC, Knauf VC (1988), Transformation of Brassica napus using Agrobacterium tumefaciens – Developmentally regulated Expression of a reintroduced napin gene. TAG, 75: (5) 685-694 .

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Stålberg K, Ellerstöm M, Ezcurra I, Ablov S, Rask L (1996). Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic Brassica napus seeds. Planta 199(4):515-9.

SEQUENCE LISTING

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+ choroplast localization signal + beta-carotene C-4 oxygenase
coding sequence + termination sequence

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cagatggcag aaatgtatca accaatgcat atatacaaat gtacctcttg ttctcaaaac 240

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Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala Phe Pro Ala Thr Arg
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        40                  45                  50

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    55                  60                  65

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70                  75                  80                  85

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            105                 110                 115

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    50                  55                  60

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                85                  90                  95

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Ser Gly Arg Gly Leu Val Pro Ala Leu Ala
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## Claims

1. A DNA construct comprising in the 5′ to 3′ direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

2. The DNA construct according to claim 1, which between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity additionally comprises a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

3. The DNA construct according to claim 1 or 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, β-carotene C-4-oxygenase, and acyl transferase activity.

4. The DNA construct according to any one of claims 1 - 3, wherein the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β-carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

5. The DNA construct according to claim 4, wherein the nucleotide sequence is SEQ ID NO:1.

6. Transgenic oilseed plant cell comprising the DNA construct of any one of claims 1-5 .

7. Transgenic oilseed plant cell according to claim 6, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

8. Transgenic oilseed plant-produced xanthophyll.

9. Transgenic oilseed plant-produced canthaxanthin

10. Transgenic oilseed plant-produced astaxanthin.

11. Transgenic oilseed plant-produced astaxanthin esters.

Napin promoter

```
AAGCTTTCTTCATCGGTGATTGATTCCTTTAAAGACTTATGTTTCTTATCTTGCTTCTGA

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ACATTAGCTACACGTTACACATAGCATGCAGCCGCGGAGAATTGTTTTTCTTCGCCACTT

GTCACTCCCTTCAAACACCTAAGAGCTTCTCTCTCACAGCACACACATACAATCACATGC

GTGCATGCATTATTACACGTGATCGCCATGCAAATCTCCTTTATAGCCTATAAATTAACT

CATCCGCTTCACTCTTTACTCAAACCAAAACTCATCAATACAAACAAGATTAAAAACATA
```

End -28 untranslated leader TP start

```
CACGAGGATCCTCAGTCACACAAAGAGTAAAGAAGAACAATGGCTTCCTCTATGCTCTCT
                                          M  A  S  S  M  L  S

TCCGCTACTATGGTTGCCTCTCCGGCTCAGGCCACTATGGTCGCTCCTTTCAACGGACTT
 S  A  T  M  V  A  S  P  A  Q  A  T  M  V  A  P  F  N  G  L

AAGTCCTCCGCTGCCTTCCCAGCCACCCGCAAGGCTAACAACGACATTACTTCCATCACA
 K  S  S  A  A  F  P  A  T  R  K  A  N  N  D  I  T  S  I  T
```

FIG.1

TP End C-4-Oxygenase

```
AGCAACGGCGGACGCGTTAACTGCATGTCTAGAATGCCATCCGAGTCGTCAGACGCAGCT
 S  N  G  G  R  V  N  C  M  S  R  M  P  S  E  S  S  D  A  A

CGTCCTGCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATG
 R  P  A  L  K  H  A  Y  K  P  P  A  S  D  A  K  G  I  T  M

GCGCTGACCATCATTGGCACCTGGACCGCAGTGTTTTTACACGCAATATTTCAAATCAGG
 A  L  T  I  I  G  T  W  T  A  V  F  L  H  A  I  F  Q  I  R

CTACCGACATCCATGGACCAGCTTCACTGGTTGCCTGTGTCCGAAGCCACAGCCCAGCTT
 L  P  T  S  M  D  Q  L  H  W  L  P  V  S  E  A  T  A  Q  L

TTGGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGTACTTGAGTTCCTG
 L  G  G  S  S  S  L  L  H  I  A  A  V  F  I  V  L  E  F  L

TACACTGGTCTATTCATCACCACACATGACGCAATGCATGGCACCATAGCTTTGAGGCAC
 Y  T  G  L  F  I  T  T  H  D  A  M  H  G  T  I  A  L  R  H

AGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACTGTACGCCTGGTTTGACTAC
 R  Q  L  N  D  L  L  G  N  I  C  I  S  L  Y  A  W  F  D  Y

AGCATGCTGCATCGCAAGCACTGGGAGCACCACAACCATACTGGCGAAGTGGGGAAAGAC
 S  M  L  H  R  K  H  W  E  H  H  N  H  T  G  E  V  G  K  D

CCTGACTTCCACAAGGGAAATCCCGGCCTTGTCCCCTGGTTCGCCAGCTTCATGTCCAGC
 P  D  F  H  K  G  N  P  G  L  V  P  W  F  A  S  F  M  S  S

TACATGTCCCTGTGGCAGTTTGCCCGGCTGGCATGGTGGGCAGTGGTGATGCAAATGCTG
 Y  M  S  L  W  Q  F  A  R  L  A  W  W  A  V  V  M  Q  M  L

GGGGCGCCCATGGCAAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTCAGCATTC
 G  A  P  M  A  N  L  L  V  F  M  A  A  A  P  I  L  S  A  F

CGCCTCTTCTACTTCGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCAGGC
 R  L  F  Y  F  G  T  Y  L  P  H  K  P  E  P  G  P  A  A  G

TCTCAGGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGTTTC
 S  Q  V  M  A  W  F  R  A  K  T  S  E  A  S  D  V  M  S  F

CTGACATGCTACCACTTTGACCTGCACTGGGAGCACCACAGATGGCCCTTTGCCCCCTGG
 L  T  C  Y  H  F  D  L  H  W  E  H  H  R  W  P  F  A  P  W
```

C-4 oxygenase Stop

```
TGGCAGCTGCCCCACTGCCGCCGCCTGTCCGGGCGTGGCCTGGTGCCTGCCTTGGCATGA
 W  Q  L  P  H  C  R  R  L  S  G  R  G  L  V  P  A  L  A  *
```

FIG.1 (cont.)

C-4 oxygenase untranslated region Nos term

CCTGGTCCCTCCGCTGGTGACCCAGCGTCTGCACAAGAGTGTCATGGAGCTCGAATTTCC

CCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTG

CGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAAT

GCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAAT

ACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCAT

end

CTATGTTACTAGATCGGGAATTC

Fig.1 (cont.)

29295/BN

## Abstract

A DNA construct comprising in the 5′ to 3′ direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme activity is preferably a peptide with β-carotene C-4-oxygenase activity, e.g. from the alga *Haematococcus pluvialis*.

Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin; transgenic oilseed plant-produced xanthophyll; transgenic oilseed plant-produced canthaxanthin; transgenic oilseed plant-produced astaxanthin; and transgenic oilseed plant-produced astaxanthin esters.